EXHIBIT A

Currently pending claims of U.S. Ser. No. 08/051,455

30. [Amended] A method of modulating interaction between a bone marrow stromal cell and a bone marrow cell which comprises administering an antibody to VCAM-1 in an amount effective to decrease adhesion between the bone marrow stromal cell and the bone marrow cell.

--31. The method of claim 30 wherein the antibody to VCAM-1 is selected from the group consisting of monoclonal antibodies and antigen-binding fragments of said monoclonal antibodies that specifically bind to an epitope recognized by 6G10 monoclonal antibody produced by hybridoma ATCC No. HB 10519.

32. The method of claim 30 wherein the bone marrow cell expresses CD34 antigen.

33. The method of claim 30 wherein the bone marrow cell is a stem cell or a progenitor cell.--

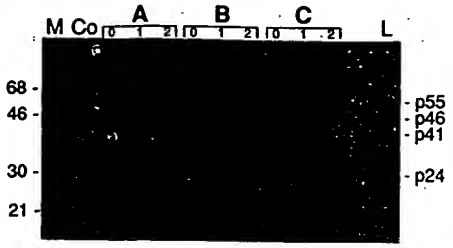

Fig. 4. U-81749 inhibition of HIV-1–like particle maturation is partially reversible. HIV-1–like particles were recovered from the medium of vVK-1–infected CV-1 cells as described in the legend to Fig. 3. The particulate fractions were resuspended in PBS with or without 10 μM U-81749 (lanes 0) and then incubated at 37°C for 1 or 2 hours (lanes 1 and 2, respectively). Samples were taken for protein immunoblot analysis with sera containing antibodies to p24 as described in Fig. 2. (**A**) Particulate fraction from infected cultures exposed to 10 μM drug and incubated with the drug. (**B**) Particulate fraction from infected cultures exposed to 10 μM drug and subsequently incubated in the absence of drug. (**C**) Particulate fraction from cultures without drug and incubated without drug. Lane Co, particulate fraction from uninfected cultures; lane L, control HIV-1 lysate indicating p24 (Scripps Laboratories, San Diego, California); and lane M, standard protein molecular weight markers.

anti-HIV activity in H9 cells at the relatively high concentration of 100 μM (*26*). In this report, we present the structure of U-81749, a synthetic compound specifically designed to inhibit the HIV-1 protease which exhibits potent anti-HIV activity in acutely infected PBLs (ED_{50} 0.1 to 1 μM). Our data suggest that U-81749 inhibits protease activity in these cells, resulting in an inhibition of viral maturation and a reduction in the levels of infectious virus. These findings provide evidence that a chemical compound, specifically targeted against the HIV-1 protease, can inhibit viral replication in cell culture. Although the observed reversibility of U-81749's protease inhibition might be expected to reduce or preclude its anti-HIV activity in vivo, the general nontoxic nature of this type of peptidemimetic compound to animals (*27*) suggests that nontoxic U-81749 derivatives with potentially irreversible protease inhibition might be obtained. Taken together, the above data support the concept of the protease as an attractive target for the development of anti-HIV agents.

REFERENCES AND NOTES

1. L. Ratner *et al.*, *Nature* 313, 277 (1985).
2. S. Wain-Hobson *et al.*, *Cell* 40, 9 (1985).
3. L. H. Pearl and W. R. Taylor, *Nature* 329, 351 (1987).
4. T. Jacks *et al.*, *ibid.* 331, 280 (1988).
5. M. A. Navia *et al.*, *ibid.* 337, 615 (1989).
6. A. Wlodawer *et al.*, *Science* 245, 616 (1989).
7. S. Seelmeier, H. Schmidt, V. Turk, K. von der Helm, *Proc. Natl. Acad. Sci. U.S.A.* 85, 6612 (1988).
8. J. Schneider and S. B. H. Kent, *Cell* 54, 363 (1988).
9. R. F. Nutt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 85, 7129 (1988).
10. T. D. Meek *et al.*, *ibid.* 86, 1841 (1989).
11. P. L. Darke *et al.*, *J. Biol. Chem.* 264, 2307 (1989).
12. S. Broder, in *Human Retroviruses, Cancer, and AIDS*, D. Bolognesi, Ed. (Liss, New York, 1988), p. 365.
13. D. D. Richman *et al.*, *N. Engl. J. Med.* 317, 192 (1987).
14. J. D. Lifson *et al.*, *Science* 241, 712 (1988).
15. P. L. Nara, K. M. Hwang, D. M. Rausch, J. D. Lifson, L. E. Eiden, *Proc. Natl. Acad. Sci. U.S.A.* 86, 7139 (1989).
16. A. D. Richards *et al.*, *FEBS Lett.* 247, 113 (1989).
17. A. G. Tomasselli *et al.*, *Biochemistry* 29, 264 (1990).
18. The HIV infectivity experiments were conducted in primary cultures of human peripheral blood lymphocytes (PBLs) with HTLV-IIIB. Cultures were infected in triplicate with HIV, U-81749 added, and 3 to 4 days after infection the levels of HIV p24 synthesized and released were quantified by an enzyme-linked immunosorbent assay (ELISA) [R. Maiolini and R. Masseyeff, *J. Immunol. Methods* 8, 223 (1975)] using a monoclonal capture antibody to HIV p24 (DuPont, Wilmington, DE) and recombinant HIV p24 calibration standard (MicroGenSys, West Haven, CT). The amounts of HIV RNA synthesized in these infected cultures were also determined by hybridization analysis with a HIV-specific ^{-32}P-labeled probe. The absolute HIV RNA levels were determined by normalizing hybridization values to values obtained from a standard preparation of HIV RNA hybridized in parallel.
19. The proliferation of mitogen-stimulated PBLs 4 days after exposure to U-81749 was quantified relative to nondrug-treated controls to ensure that the infectivity experiments were conducted at noncytotoxic concentrations of drug. At 1 μM U-81749 did not significantly inhibit PBL proliferation (<10% inhibition) and proliferation was reduced <40% at 10 μM.
20. V. Karacostas, K. Nagashima, M. Gonda, B. Moss, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
21. N. E. Kohl *et al.*, *ibid.* 85, 4686 (1988).
22. M. C. Graves *et al.*, *ibid.*, p. 2449.
23. C. Debouck *et al.*, *ibid.* 84, 8903 (1987).
24. J. Mous *et al.*, *J. Virol.* 62, 1433 (1988).
25. T. D. Copeland and S. Oroszlan, *Gene Anal. Tech.* 5, 109 (1988).
26. K. von der Helm *et al.*, *FEBS Lett.* 247, 349 (1989).
27. T. J. Kakuk, personal communication.

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Intercellular Adhesion Molecule–1 (ICAM-1) in the Pathogenesis of Asthma

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Airway eosinophilia, epithelial desquamation, and hyperresponsiveness are characteristics of the airway inflammation underlying bronchial asthma. The contribution of intercellular adhesion molecule–1 (ICAM-1) to eosinophil migration and airway responsiveness was studied. ICAM-1 partially mediated eosinophil adhesion to endothelium in vitro and was upregulated on inflamed bronchial endothelium in vivo. ICAM-1 expression was also upregulated on inflamed airway epithelium in vitro and in vivo. In a primate model of asthma, a monoclonal antibody to ICAM-1 attenuated airway eosinophilia and hyperresponsiveness. Thus, antagonism of ICAM-1 may provide a therapeutic approach to reducing airway inflammation, hyperresponsiveness, and asthma symptoms.

A MAJOR CHARACTERISTIC OF ASTHMA is the extreme (10 to 1000 times normal) sensitivity of the bronchi to inhaled agents (*1, 2*). The severity of this "airway hyperresponsiveness" correlates with the intensity of asthmatic symptoms (*2–4*), diurnal variations in airway caliber (*5*), and therapy required (*2, 6*). Although the underlying pathogenetic mechanisms are not known, many studies suggest that eosinophil infiltration and desquamation of the bronchial epithelium are involved (*7, 8*). Since eosinophil-derived mediators damage airway epithelial cells in vitro, these two events may be linked (*9*). We investigated a mechanism by which eosinophils enter the airways and contribute to airway hyperresponsiveness in primates.

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Adhesion of leukocytes to microvascular endothelium is essential for their migration into inflamed tissues. This was demonstrated in a group of patients who, because of an inherited genetic defect, are deficient in the expression of the CD18 family of leukocyte adhesion receptors (*10*). Infected tissues in severely affected patients contain little or no neutrophils. A ligand for some (*11, 12*) of these adhesion receptors is intercellular adhesion molecule–1 (ICAM-1). ICAM-1 was shown to be upregulated on endothelium and skin epithelium both in vitro and in vivo 4 to 24 hours after an inflammatory stimulus (*13–15*). In addition, monoclonal antibodies (MAbs) to ICAM-1 attenuate neutrophil adhesion to endothelium and inhibit neutrophil transendothelial migration in vitro (*12, 15*) and in vivo (*16*). Thus, ICAM-1 appears to be required for neutrophil migration into inflamed tissues.

In this report, we investigated (i) the

contribution of ICAM-1 to eosinophil adhesion to endothelium in vitro; (ii) the upregulation of ICAM-1 on airway epithelium in vitro and in vivo, as well as on bronchial vascular endothelium in vivo; and (iii) the contribution of ICAM-1 to the eosinophil infiltration and increase in airway responsiveness induced by multiple inhalations of antigen in vivo.

The adhesion of primate lung eosinophils (stimulated with platelet-activating factor) to a monolayer of human umbilical vein endothelial cells (HUVECs, previously stimulated with lipopolysaccharide) was inhibited by the MAb RR1/1 to ICAM-1 (Fig. 1A). In contrast, the control MAb W6/32 to human lymphocyte antigen (HLA) class I, which also binds to HUVECs (*15*), did not inhibit eosinophil adhesion. Adhesion of primate lung eosinophils to immune complex–coated plastic was not inhibited by RR1/1 (Fig. 1B), which demonstrated the specificity of the inhibition of adherence to endothelium. Thus, ICAM-1 is important for eosinophil adhesion to endothelium and may contribute to eosinophil migration into inflamed tissue in vivo.

Leukocyte adhesion is also a likely prerequisite for cytotoxic tissue injury (*17*). Thus, with an enzyme-linked immunosorbent assay (ELISA) we investigated the abilities of inflammatory cytokines to enhance ICAM-1 expression on a monolayer of cultured monkey bronchus epithelial cells. Interleukin-1β (IL-1β), human recombinant tumor necrosis factor α (TNFα), and most markedly human recombinant interferon-γ (IFN-γ) enhanced ICAM-1 expression in a concentration-dependent manner (Table 1). As expected, the MAb R3.1 to CD11a did not bind to unstimulated or stimulated epithelial cells. The time course of enhanced ICAM-1 expression [onset ≥2 hours and peak at 12 to 16 hours (*18*)] was similar to that reported for HUVECs in vitro (*15*) and human skin keratinocytes in vivo (*14*). Interestingly, viral infections, which stimulate an inflammatory immune response including interferon production, are a common initiator-enhancer of airway hyperresponsiveness and asthma symptoms (*19*). Finally, in preliminary studies, adhesion of monkey lung eosinophils to a monolayer of monkey airway

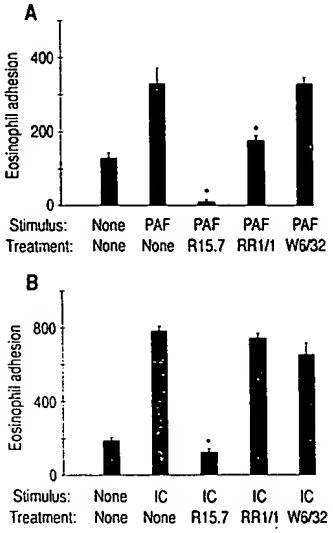

Fig. 1. Effects of MAbs (1:4 dilution of supernatants) RR1/1 to ICAM-1 (*11*), R15.7 to CD18, and W6/32 to HLA class I (*15*) on (**A**) platelet-activating factor (PAF) ($10^{-7}M$) induced eosinophil adhesion to lipopolysaccharide (LPS) (10 ng/ml) stimulated human umbilical vein endothelium, and (**B**) eosinophil adhesion to flat-bottom tissue culture plate wells coated with *Ascaris* extract (no stimulus) or immune complex (IC stimulus). Eosinophils were obtained by bronchoalveolar lavage from adult male cynomolgus monkeys (*Macaca fascicularis*) with airway eosinophilia, purified (morphologically >95% pure) on a Percoll continuous-density gradient (*26*), washed, and added to 96-well flat-bottom tissue culture plates (5×10^3 cells per well). After a 60-min incubation at 37°C, the nonadherent cells were removed by an automated plate washer. Adherent cells were quantitated visually and by a colorimetric assay for eosinophil peroxidase (EPO) (*27*). Human umbilical vein endothelial cells were isolated, cultured to a confluent monolayer in each well, stimulated for 4 hours with LPS, and fixed in 1% glutaraldehyde (*15*). Immune complex wells were made by coating with *Ascaris* extract and then adding serum from an *Ascaris*-sensitive monkey. Bars are mean + SEM for three to six wells and are representative of more than six individual experiments. Significant inhibition (*, $P < 0.05$) of stimulated adhesion was determined by comparison of RR1/1 and R15.7 treatment to W6/32 or nontreatment and calculated with both Dunnett's and Tukey's multiple comparison tests.

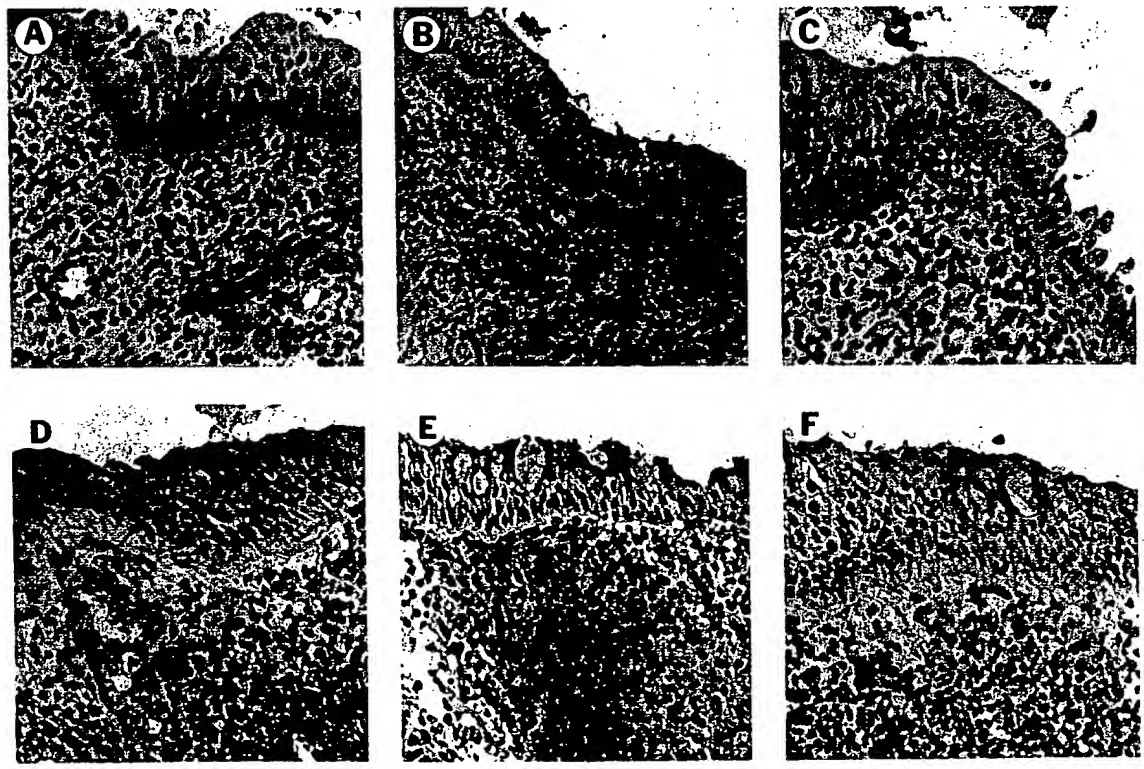

Fig. 2. Immunohistochemical staining with (**A**) antibody to ICAM-1 (anti–ICAM-1), (**B**) antibody to CD11a (anti-CD11a), and (**C**) normal mouse serum in a trachea section taken from an *Ascaris*-sensitive cynomolgus monkey 20 min after the third of three alternate-day *Ascaris* inhalations. For comparison, staining with (**D**) anti–ICAM-1, (**E**) anti-CD11a, and (**F**) normal mouse serum in a trachea section taken from an *Ascaris*-sensitive monkey 20 min after a single *Ascaris* inhalation. Tissues were stained with a modification of a described protocol (*14*). Briefly, tissue specimens were removed and frozen in liquid nitrogen. After cryo-sectioning, 5- to 10-μm sections were fixed in acetone for 10 min and either stained immediately or stored at −20°C. Staining was done with the Biotin-Strept Avidin System kit according to manufacture's protocols (BioGenex). Primary antibody was incubated with tissue as undiluted culture supernatants (RPMI 1640 medium with 10% fetal bovine serum) for 1 hour at room temperature. Blocking for nonspecific protein binding was accomplished by applying normal goat serum. We used 3-amino-9-ethylcarbazole (AEC) as a substrate and the sections were counterstained with Mayer's hematoxylin.

epithelial cells was largely inhibited by the MAb R6.5 to ICAM-1 (*18*). Thus, by mediating close contact between eosinophils and inflamed airway epithelium, ICAM-1 may contribute to eosinophil-induced desquamation of airway epithelium in vivo.

Table 1. Effects of inflammatory cytokines on ICAM-1 expression on bronchial epithelial cells in vitro. The rhesus monkey bronchus epithelial cell line 4MBr-5 (obtained from American Type Culture Collection) was cultured to a confluent monolayer and then stimulated for 16 hours with IL-1β, TNFα, or IFN-γ. ELISA assays for ICAM-1 [MAbs RR1/1 (*11*) and R6.5 (*15*)] and CD11a [MAb R3.1 (*25*)] were as described (*25*). The numbers are the mean optical density units (relative to normal mouse gamma globulin background) for duplicate cultures and are representative of four individual experiments.

Stimulus	Concentration (unit/ml)	Optical density units		
		RR1/1 (anti–ICAM-1)	R6.5 (anti–ICAM-1)	R3.1 (anti-CD11a)
None		92	206	−27
IL-1β	0.1	130	253	−33
	1	166	324	−32
	10	149	322	−37
TNFα	1	100	236	−30
	10	131	266	−29
	100	159	346	−31
	1000	178	416	−36
IFN-γ	0.1	138	276	−16
	1	263	423	−17
	10	413	673	−26
	100	576	940	−27

We then used immunohistochemical staining to determine if multiple inhalations of antigen enhanced ICAM-1 expression on airway epithelium in vivo. ICAM-1 intensely stained on both the epithelium (basilateral portion only) and on the vascular endothelium of a trachea section taken from a monkey 20 min after the third of three alternate-day antigen inhalations (Fig. 2A). Staining for CD11a [a receptor for ICAM-1 on most leukocytes (*10*), but not on airway epithelium (Table 1)] revealed a leukocyte infiltration in the interstitium, particularly just below the epithelial basement membrane (Fig. 2B). Leukocytes were also between epithelial cells, primarily at the basilateral portion of the epithelium, where ICAM-1 staining was most pronounced. Normal mouse serum showed little or no nonspecific staining (Fig. 2C). For comparison, Fig. 2, D, E, and F, show ICAM-1, CD11a, and normal mouse serum, respectively, staining in a trachea section removed 20 min after a single inhalation of antigen. As expected, on the basis of the time required for its expression (*14, 15, 18*), little ICAM-1 staining was found on the epithelium or vascular endothelium in this section. In addition, although there were pockets of leukocyte infiltration, leukocytes were not accumulated just below the epithelial basement membrane or between epithelial cells.

Three alternate-day inhalations of antigen induce a consistent (usually greater than eightfold) increase in airway responsiveness to inhaled methacholine in monkeys (*20*). This increase in airway responsiveness is preceded by an intense eosinophil infiltration and is similar in magnitude to that induced in asthmatics during the pollen season (*3, 21*) or to continued exposure to occupational allergens (*4, 22*). In this animal model, daily intravenous treatment with the MAb R6.5 to ICAM-1 attenuated the eosinophil infiltration in the five animals studied (Fig. 3A). The increase in airway responsiveness [decrease in inhaled methacholine PC_{100} (*20*)] was also inhibited in the five animals, markedly in four (Fig. 3B). Surprisingly, in two animals (c and d) airway responsiveness actually decreased (methacholine PC_{100} increased) after R6.5 treatment, despite the multiple inhalations of antigen, demonstrating a reversal of an elevated basal airway responsiveness in these animals. Thus, antagonism of ICAM-1 attenuates eosinophil infiltration and (more importantly) the induction airway hyperresponsiveness in vivo.

These results indicate that ICAM-1 may be pivotal in the pathogenesis of airway hyperresponsiveness and asthma. By inference, ICAM-1 may also contribute to the onset and progression of other diseases characterized by airway inflammation [for example, chronic bronchitis, emphysema, and idiopathic pulmonary fibrosis (*23*)] or eosinophil infiltration and tissue sensitization-destruction [for example, rhinitis, nasal polyposis, chronic urticaria, and atopic dermatitis (*24*)].

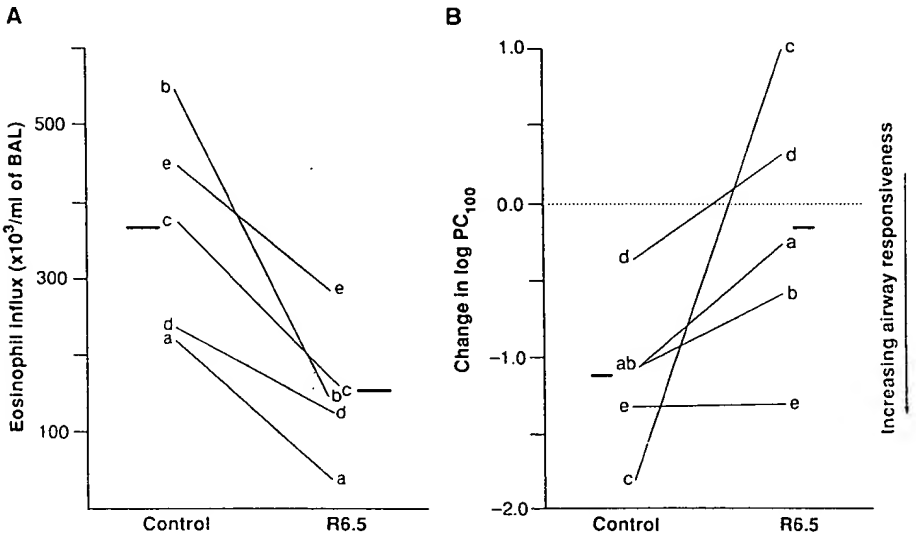

Fig. 3. Effect of the MAb R6.5 to ICAM-1 on the (**A**) airway eosinophil infiltration and (**B**) increase in airway responsiveness [decrease in methacholine PC_{100} (provocation concentration of inhaled methacholine to produce a 100% increase in respiratory system resistance)] induced by multiple inhalations of antigen in monkeys. Studies with R6.5 treatment are compared to the mean of bracketing control studies done on each animal (*20*). The solid bars represent the mean of the five animals individually identified by the letters a to e. See (*20*) for protocol and methods. R6.5 treatment significantly attenuated the eosinophil influx when tested versus the bracketing control values by two-way analysis of variance ($P = 0.0113$) and versus the post-R6.5 control study by Friedman's test ($P = 0.0212$); Friedman's for R6.5 versus the pre-R6.5 control was $P = 0.0755$. R6.5 treatment significantly inhibited the decrease in log PC_{100} versus the pre-R6.5 control by Friedman's test ($P = 0.0002$); Friedman's for R6.5 versus the post-R6.5 control was $P = 0.0667$. BAL, bronchoalveolar lavage.

REFERENCES AND NOTES

1. H. A. Boushey, M. J. Holtzman, J. R. Sheller, J. A. Nadel, *Am. Rev. Respir. Dis.* **121**, 389 (1980).
2. F. E. Hargreave *et al.*, *J. Allergy Clin. Immunol.* 68, 347 (1981).
3. L.-P. Boulet *et al.*, *ibid.* **71**, 399 (1983).
4. M. Chan-Yeung, S. Lam, S. Koener, *Am. J. Med.* 72, 411 (1982).
5. G. Ryan *et al.*, *Thorax* 37, 423 (1982).
6. E. F. Juniper, P. A. Frith, F. E. Hargreave, *ibid.* 36, 575 (1981).
7. J. G. R. DeMonchy *et al.*, *Am. Rev. Respir. Dis.* 131, 373 (1985); S. R. Durham and A. B. Kay, *Clin. Allergy* 15, 411 (1985).
8. L. A. Laitinen, M. Heino, A. Laitinen, T. Kava, T. Haahtela, *Am. Rev. Respir. Dis.* 131, 599 (1985); A. J. Wardlaw, S. Dunnette, G. J. Gleich, J. V. Collins, A. B. Kay, *ibid.* 137, 62 (1988).
9. E. Frigas and G. J. Gleich, *J. Allergy Clin. Immunol.* 77, 527 (1986).
10. D. C. Anderson *et al.*, *J. Inf. Dis.* 152, 668 (1985); D. C. Anderson and T. A. Springer, *Annu. Rev. Med.* 38, 175 (1987); R. F. Todd and D. R. Freyer, *Hematol. Oncol. Clin. North Am.* 2, 13 (1988).
11. R. Rothlein, M. L. Dustin, S. D. Marlin, T. A. Springer, *J. Immunol.* 137, 1270 (1986); S. D. Marlin and T. A. Springer, *Cell* 51, 813 (1987).
12. C. W. Smith *et al.*, in *Structure and Function of Molecules Involved in Leukocyte Adhesion*, T. A. Springer, D. C. Anderson, A. S. Rosenthal, R. Rothlein, Eds. (Springer-Verlag, New York, 1989).
13. M. L. Dustin, R. Rothlein, A. K. Bhan, C. A. Dinarello, T. A. Springer, *J. Immunol.* 137, 245 (1986); M. L. Dustin, K. H. Singer, D. T. Tuck, T. A. Springer, *J. Exp. Med.* 167, 1323 (1988).
14. G. L. Wantzin *et al.*, *J. Am. Acad. Dermatol.* 20, 782 (1989).

15. C. W. Smith *et al.*, *J. Clin. Invest.* **82**, 1746 (1988).
16. R. W. Barton, R. Rothlein, J. Ksiazek, C. Kennedy, *J. Immunol.* **143**, 1278 (1989).
17. E. Martz and S. H. Gromkowski, in *Mechanisms of Cell-Mediated Cytotoxicity II*, P. Henkart and E. Martz, Eds. (Plenum Press, New York, 1985), pp. 1–17; S. Kohl, T. A. Springer, F. C. Schmalstieg, L. S. Loo, D. C. Anderson, *J. Immunol.* **133**, 2972 (1984).
18. C. D. Wegner and P. Reilly, unpublished data.
19. D. W. Empey, L. A. Laitinen, L. Jacobs, W. M. Gold, J. A. Nadel, *Am. Rev. Respir. Dis.* **113**, 131 (1976); C. B. Sherter and C. A. Polnitsky, *Clin. Chest Med.* **2**, 67 (1981).
20. As described [C. D. Wegner, C. A. Torcellini, C. C. Clarke, R. H. Gundel, *Am. Rev. Respir. Dis.* **139**, A324 (1989)], airway cell composition and airway responsiveness were determined 3 days before (day 0) and 3 days after (day 10) three alternate-day (day 3, 5, and 7) inhalations of *Ascaris* in *Ascaris*-sensitive cynomolgus monkeys. Airway cell composition was measured by bronchoalveolar lavage and airway responsiveness as the concentration (PC_{100}) of inhaled methacholine that caused a 100% increase in respiratory system resistance, an estimation of airway caliber [R. H. Gundel, M. E. Gerritsen, C. D. Wegner, *Am. Rev. Respir. Dis.* **140**, 629 (1989)]. MAb R6.5 was administered intravenously at 1.76 mg per kilogram of body weight daily on days 2 to 9. Studies with R6.5 treatment were compared to the mean of bracketing control studies for each animal to compensate for any change in sensitivity-reactivity of an animal. Studies on each animal were separated by 5 weeks to allow airway inflammation and responsiveness to return to baseline (before antigen) levels.
21. H. Sotomayor, M. Badier, D. Vervloet, J. Orehek, *Am. Rev. Respir. Dis.* **130**, 56 (1984).
22. S. Lam, F. Tan, H. Chan, M. Chan-Yeung, *J. Allergy Clin. Immunol.* **72**, 134 (1983); S. Lam, R. Wong, M. Yeung, *ibid.* **63**, 28 (1979).
23. C. A. Guenter, J. J. Coalson, J. Jacques, *Am. Rev. Respir. Dis.* **123**, 79 (1981); G. A. Rossi *et al.*, *ibid.* **129**, 850 (1984); G. W. Hunninghake *et al.*, *ibid.* **123**, 407 (1981); G. W. Hunninghake, J. E. Gadek, O. Kawanami, V. J. Ferrans, R. G. Crystal, *Am. J. Pathol.* **97**, 149 (1979).
24. N. Mygind, *Allergy* **34**, 195 (1979); M. F. Mullarkey, J. S. Hill, D. R. Webb, *J. Allergy Clin. Immunol.* **65**, 122 (1980); M. S. Peters, A. L. Schroeter, G. M. Kephart, G. J. Gleich, *J. Invest. Dermatol.* **81**, 39 (1983); K. M. Leiferman *et al.*, *N. Engl. J. Med.* **313**, 282 (1985); C. J. F. Spry, P.-C. Tai, J. Barkans, *Int. Arch. Allergy Appl. Immunol.* **77**, 252 (1985).
25. R. Rothlein *et al.*, *J. Immunol.* **141**, 1665 (1988).
26. G. A. Riding and P. Willadsen, *J. Immunol. Methods* **46**, 113 (1981).
27. M. Strath, D. J. Warren, C. J. Sanderson, *ibid.* **83**, 209 (1985).

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The Response of Living Cells to Very Weak Electric Fields: The Thermal Noise Limit

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A physical model in which cells are considered as possible detectors of very weak periodic electric fields yields a general relation between cell size and both thermally induced fluctuations in membrane potential and the maximum change in membrane potential caused by an applied field. The simplest version of the model provides a broad-band estimate of the smallest applied electric field to which membrane macromolecules can directly respond (about 10^{-3} volt per centimeter). Much smaller fields (10^{-6} volt per centimeter) can be detected if there is a response in only a narrow band of frequencies or if signal averaging occurs through field-induced variation in the catalytic activity of membrane-associated enzymes. Both extensions of the simplest version remove the apparent violation of the thermal noise limit found in some experiments.

SIGNIFICANT BIOLOGICAL EFFECTS due to the exposure of cells to electric fields have been reported, including in vitro experiments involving isolated cells that demonstrated responses (for example, altered synthesis or transcription) to very weak fields (*1–5*). Controversy concerning the validity of weak field responses has arisen (i) because experimental reproduction of the effects has not always been achieved and (ii) because of the theoretical objection that the very small field magnitudes sometimes reported appear to be lower than allowed by thermal noise that causes randomization of cellular processes (*6*). The thermal noise limit is important because it is fundamental and also because other biological response mechanisms have a threshold close to this limit (*7*).

A cellular response to an external field implies that the applied field causes changes greater than those due to random fluctuating events ("noise"). Membranes have many sources of noise, including fundamental noise such as thermal noise, $1/f$ noise, and noise due to stochastic opening and closing of ion-conducting channels (*8*). Any cellular response to low fields must at least overcome the effects of thermal noise. Noise due to thermal fluctuations is well described by a fundamental physical theory. Random fluctuations in the transmembrane potential, $U(t)$, occur by virtue of a cell existing in a state of thermal equilibrium with its environment. As first observed by Johnson and explained by Nyquist, the thermally generated noise in an electrical resistance, R, is described by (*9*)

$$\overline{(\delta U)_{kT}^2} = 4RkT\Delta f \qquad (1)$$

where $kT = 4.3 \times 10^{-21}$ J ≈ 0.025 eV at 310 K is the product of the Boltzmann constant and the absolute temperature, Δf, is the frequency bandwidth within which information is sought, and the "bar" over $(\delta U)_{kT}^2$ denotes a time average. A worst case noise estimate can be made with a bandwidth Δf that is as large as possible (*10*). A cell membrane can be well represented by a parallel combination of membrane resistance R and membrane capacitance C, yielding an effective bandwidth $\Delta f = 1/(4RC)$ (*11*) and a broad-band estimate $\overline{(\delta U)_{kT}^2} = kT/C$.

For simplicity we first consider a spherical cell membrane of radius r_{cell}, for which the capacitance is $C \approx \epsilon_o K_m 4\pi r_{cell}^2/d$. For the broad-band case

$$\overline{(\delta U)_{kT}^2} \approx kTd/(4\pi\epsilon_0 K_m r_{cell}^2) \qquad (2)$$

where ϵ_0 ($= 8.85 \times 10^{-12}$ C/V-m) is the permittivity of free space, K_m is the membrane's dielectric constant (typically 2 to 3), and d is the membrane's electrical thickness (about 50 Å). For a typical spherical mammalian cell (10-μm radius) the estimate is $(\delta U)_{kT} \equiv \sqrt{\overline{(\delta U)_{kT}^2}} = 2.8 \times 10^{-5}$ V at $T = 310$ K as the root-mean-square (rms) variation in the transmembrane potential due to thermal fluctuations. Imposition of a narrower bandwidth results in smaller $(\delta U)_{kT}$, so that this estimate represents a worst case.

An applied field E also causes changes in U. Although the general case of nonspherical geometry and finite membrane conductance gives rise to more complicated mathematical expressions, a basic response can be illustrated for the simple case of a perfectly insulating ($\sigma = 0$) spherical shell membrane, for which (*12*)

$$\Delta U_{max} \approx 1.5 E r_{cell} \qquad (3)$$

One can obtain the simplest estimate of E_{min}, the minimum field to which a cell can respond, by comparing ΔU_{max} to $(\delta U)_{kT}$. More specifically, in order for a cell to respond to E_{min}, we estimate that the applied field, E, must induce a change, $\Delta U_{max} \approx (\delta U)_{kT}$, that corresponds to a signal-to-noise ratio (S/N) of about 1, and yields (*10*)

$$E_{min} \approx \frac{2(\delta U)_{kT}}{3 r_{cell}} = \frac{2}{3}\left[\frac{kTd}{4\pi\epsilon_0 K_m}\right]^{1/2}\frac{1}{r_{cell}^2} \equiv \frac{2A}{3}\frac{1}{r_{cell}^2} \qquad (4)$$

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